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Establishment and Maintenance of Aseptic Culture of *Trichomanes speciosum* Gametophytes from Gemmae

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ABSTRACT.—A technique is described for surface sterilization of gemmae of *Trichomanes speciosum*. The subsequent aseptic growth of gametophytes in artificial media under different temperature and light regimes is described. The utility of the technique as an alternative to surface sterilization of spores for the inception of gametophyte cultures of gemmiferous ferns and bryophytes is demonstrated.

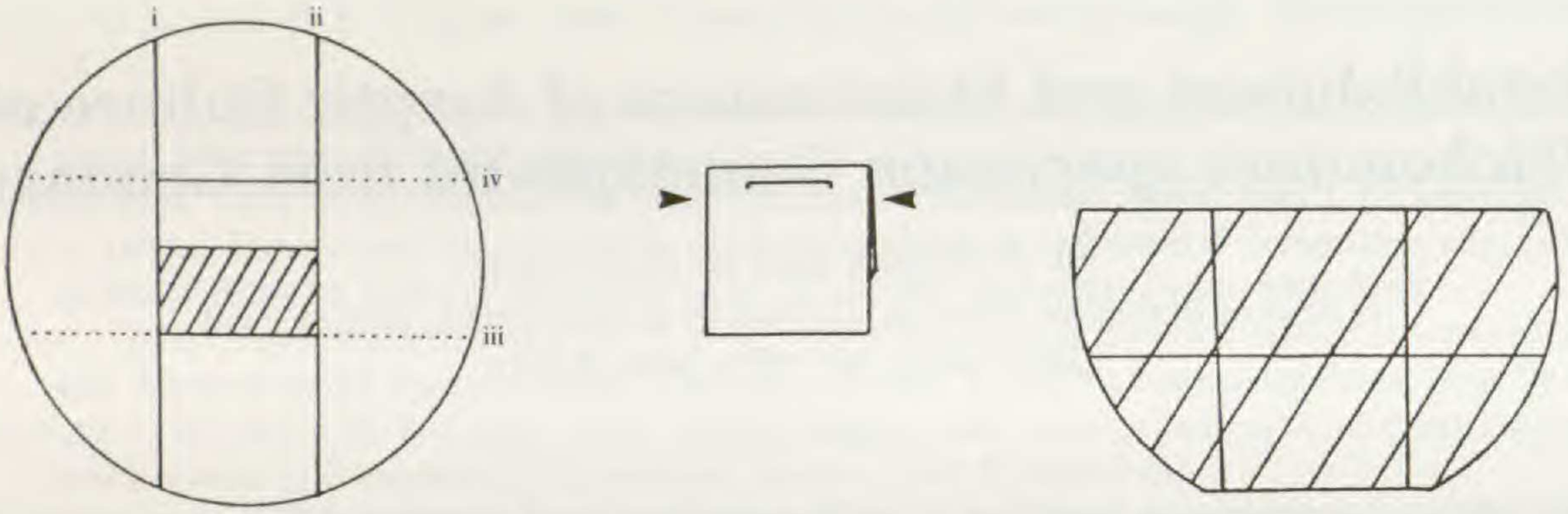
Aseptic culture of fern gametophytes is routinely initiated from surface-sterilized spores (e.g., Dyer, 1979). There are many reasons for generating such cultures, but most concern the elucidation of substrate requirements or documentation of growth and reproductive behavior in defined conditions. Gametophytes of the filmy ferns (Hymenophyllacaeae) have been cultured from spores by several workers, largely with the aim of observing their subsequent ontogeny (e.g., Stokey, 1940; Stone, 1958; Yoroi, 1972). These workers reported successful culture, but admitted to significant losses through contamination and slow germination and growth.

The Killarney fern, *Trichomanes speciosum* Willd., is one of the rarest ferns in Europe, and a licence is required for the collection of any material (Perring and Farrell, 1983). Sporophytes produce chlorophyllous spores (Stokey, 1940) very infrequently in Britain (e.g., Sheffield, 1994). The spores germinate and grow extremely slowly (Yoroi, 1972). Collection and surface-sterilization of spores of this species are therefore fraught with problems.

One way to avoid the problems caused by using spores is to use gametophytic tissue as starter material for culture. *Trichomanes speciosum* gametophytes occupy many more sites than sporophytes in Europe (e.g., Vogel et al., 1993) and gemmae are produced at most sites (Rumsey, 1994). Gemmae germinate and produce gametophytes more rapidly than do spores (Stone, 1965). The aim of this investigation was therefore to test *Trichomanes speciosum* gemmae for their suitability as material with which to establish aseptic cultures of this endangered species. Achievement of this aim necessarily involved testing the ability of various culture conditions and defined artificial media to support germination of gemmae and the subsequent growth of the filamentous gametophytes of *Trichomanes speciosum*.

MATERIALS AND METHODS

The fern spore sterilization method of Ford and Fay (1990) was refined for application to gemmae and a 0.1M solution of sodium hypochlorite low in



a b c

FIG. 1. Construction and use of filter paper packets. a) Folds necessary to generate packet; numbers indicate fold sequence; gemmae placed on hatched region only. b) Stapled packet; after sterilization the staple and top are cut off as indicated by arrows. c) Opened sterile packet, gemmae distributed throughout hatched region.

bromine (Merck Ltd., Product no. 23039) used (Adams, pers. comm.) as follows: 7cm Whatman 541 hardened ashless filter papers were folded to form filter paper packets (FPPs, see Fig. 1). Petri dishes (9 cm) were lined with filter paper and moistened with distilled water. Gemmiferous clumps of *Trichomanes speciosum* gametophyte were extracted from (soil-based) source cultures (Rumsey, 1994), inverted and dabbed over the whole of the filter paper surface. This released and dispersed gemmae. A pre-folded FPP was opened and placed in a petri dish; the central area (see hatched area on Fig. 1a) was dampened with sterilized distilled water, but not soaked. Using a dissecting microscope and fine forceps, gemmae were carefully removed from the source petri dish and placed in the central region of the open FPP. Damaged or germinated gemmae were not used; those selected consisted of 4–12 cells. Each FPP was refolded and stapled at the end furthest from the gemmae, and placed in a dish containing sterilized distilled water to avoid desiccation while others were prepared. Just before surface sterilization, the FPPs were gently squeezed to expel trapped air. Up to 18 FPPs per run were submerged in 300 ml surface sterilant in a 500ml wide-necked conical flask. FPPs were agitated in the solution via a large magnetic stirrer bar and placing on a stirrer.

The remainder of the procedure was performed in a laminar flow unit. Scissors and forceps were flame sterilized before use.

After 2, 5, or 10 min, the sterilant packets were removed one at a time using large forceps. Excess sterilant was carefully squeezed out and the packet dropped into a 500ml conical flask containing sterilized distilled water. All packets were then washed for 10 min, agitating as before. This post-sterilization procedure of removing, squeezing, and washing packets was repeated three times.

After the final wash, single packets were removed and as much water as possible expelled from the FPP. The stapled end was cut off with scissors and discarded (Fig. 1b). FPPs were opened using two pairs of small forceps. Gemmae were spread all over the exposed surface of the FPPs when opened (Fig.

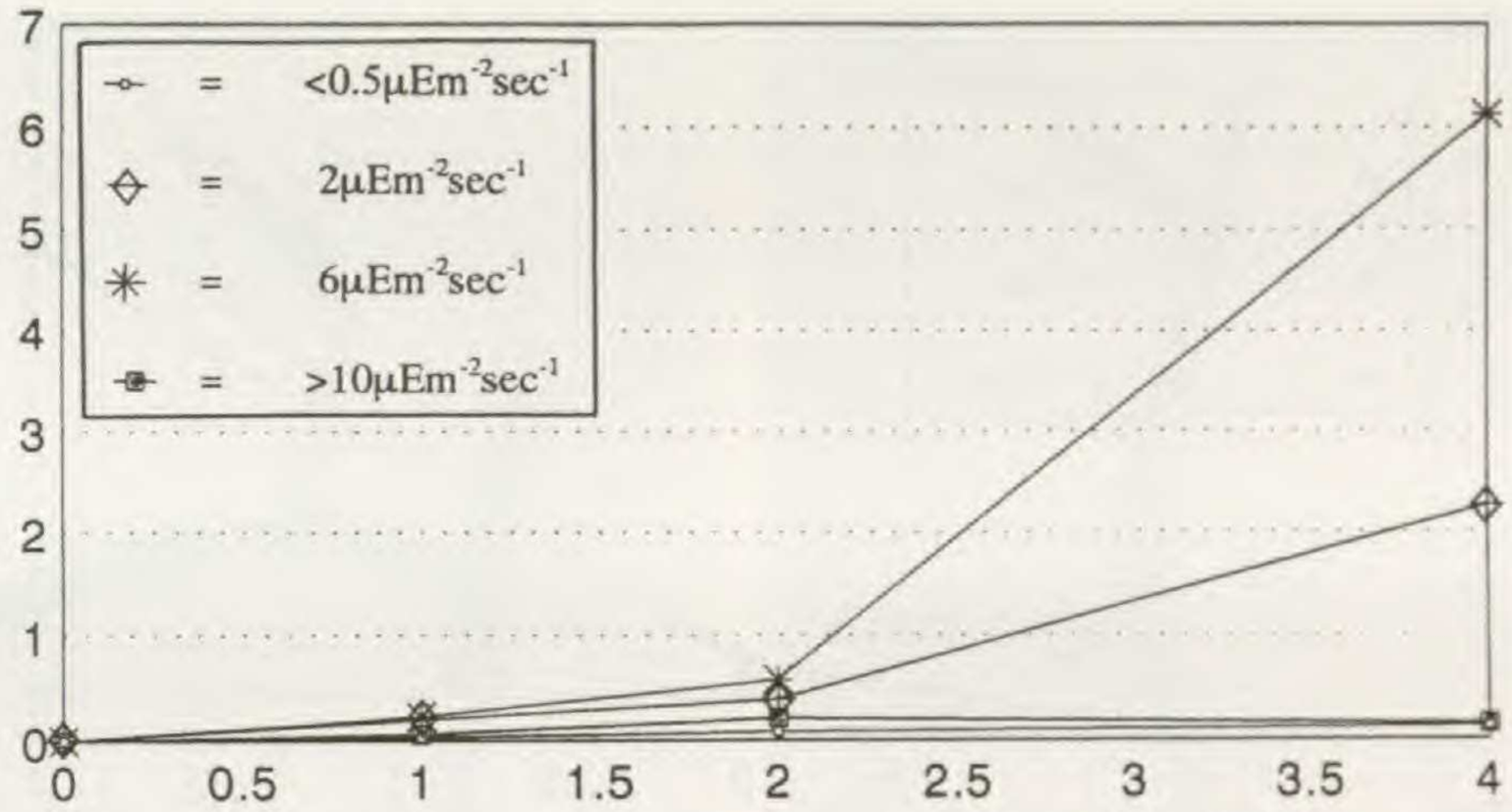


FIG. 2. Graph showing change in cell number per original cell of *Trichomanes speciosum* gemmae cultured at 12°C in 4 different PAR levels.

1c), so care was taken not to let water drop off the opened FPP, as this contained most of the gemmae. A petri dish of medium was opened, and the whole open FPP placed face down on the agar surface. The FPP was patted gently so that it was flat and in complete contact with the surface. The FPP was peeled back and forth several times to release gemmae onto the surface, and the FPP discarded. The petri dish lid was replaced, sealed with plastic film (Nescofilm), labeled, and incubated at 12° or 20°C, with constant illumination provided by fluorescent daylight tubes. Four different light intensities were generated by covering the dishes with 0, 4, 8, and 32 sheets of fine mesh muslin. Photosynthetically available radiation (PAR) values at the level of the dishes were measured.

Defined media tested included Moore's (1903) and Hoagland's (Reuter, 1953) made up at full and one tenth strength, with and without 2% sucrose (to help reveal contaminants), solidified with 0.8% Difco Bacto agar. Cultures were observed with a dissecting microscope after 1, 2, and 4 months, and the numbers of cells per original cell of all the gemmae counted (8–16 gemmae per culture).

RESULTS

Gemmae were unharmed by surface sterilization for 2 min; they germinated and grew well, but most cultures became contaminated with microorganisms. The attachment cell of each gemma died as a result of the 5 min surface sterilization procedure, but the remaining cells were unharmed and fungal contamination occurred very infrequently. A 10 min surface sterilization step reduced contamination to negligible levels, but caused the death of both terminal cells in addition to the attachment cell. A 5 min period was therefore routinely adopted for *Trichomanes speciosum* gemmae.

Gemmae were bleached and their cells became swollen at $>10\mu Em^{-2}sec^{-1}$; very little growth was observed at this level of PAR or below $0.5\mu Em^{-2}sec^{-1}$ (see Figs. 2, 3). Cultures maintained at 2 or $6\mu Em^{-2}sec^{-1}$ germinated and grew,

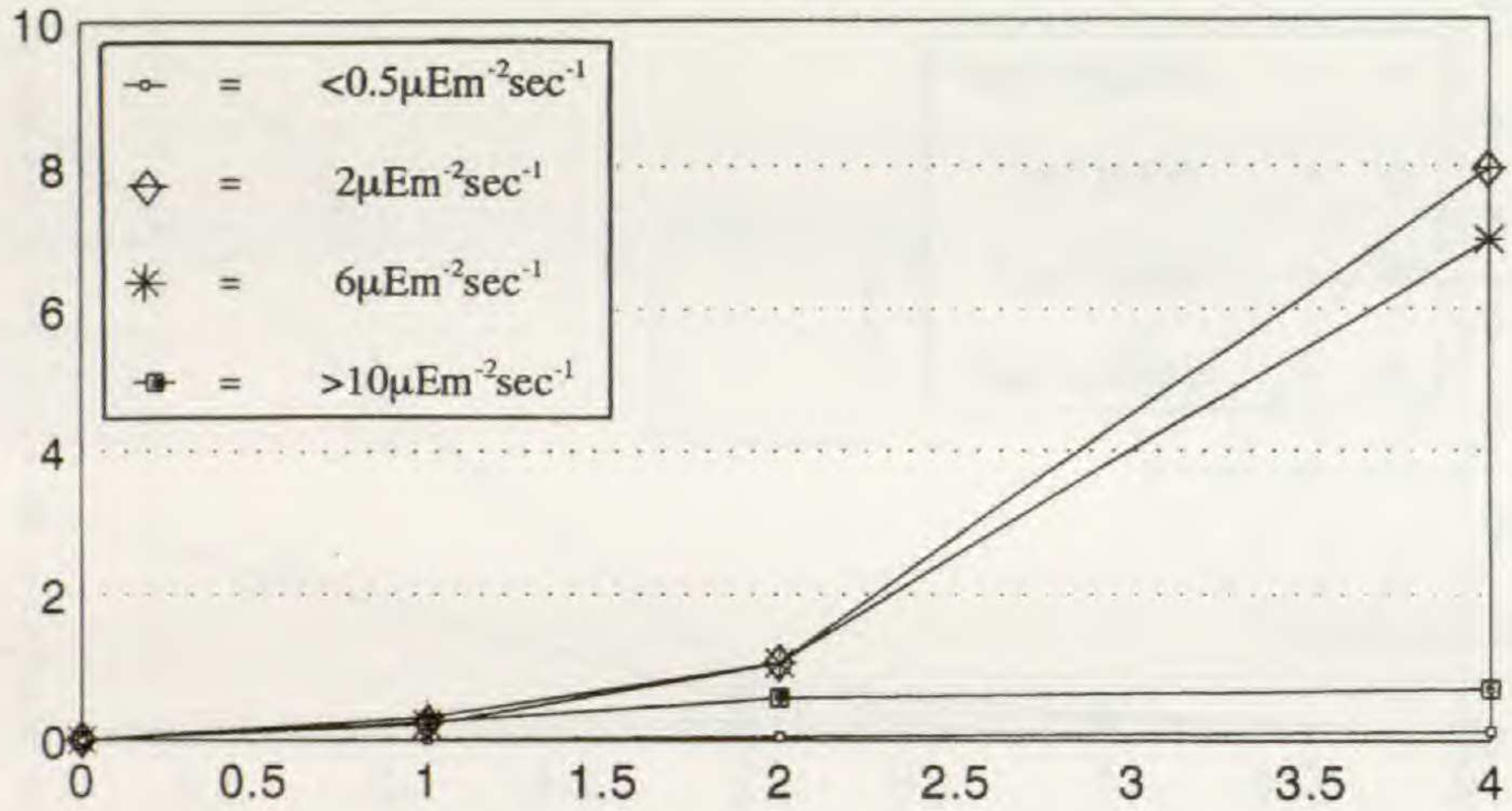


FIG. 3. Graph showing change in cell number per original cell of *Trichomanes speciosum* gemmae cultured at 20°C in 4 different PAR levels.

but those kept at 20°C produced greater biomass than those maintained at 12°C (see Figs. 2, 3).

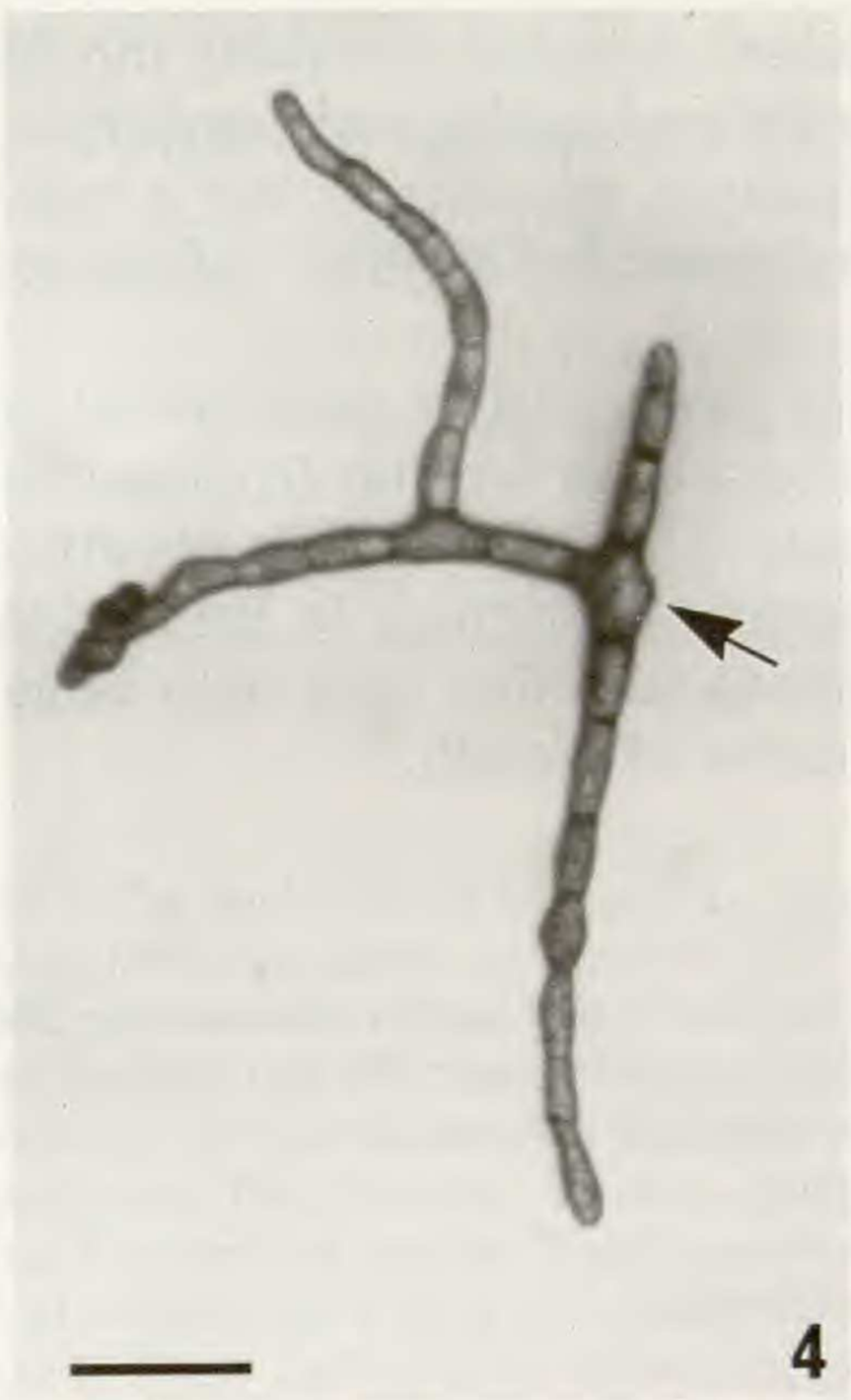
Gemmae germinated and grew on all the media tested, but one tenth strength Hoagland's with sucrose supported the most rapid growth. Gametophytes produced from gemmae generated gemmae themselves after 8 months of culture. Undisturbed cultures remained healthy and contaminant free for 50 months or more, during which time they established the three dimensional mat-like form of gametophytes seen in the wild (see Figs. 4–6).

DISCUSSION

The availability of a surface-sterilizing procedure for *Trichomanes speciosum* gemmae obviates the need for collection of spores to initiate gametophyte cultures. The method is simple, effective, and applicable to a wide range of gemmiferous plants. Although the large multicellular gemmae of the bryophyte *Tetraphis* do not survive the procedures described herein, the smaller single-celled gemmae of *Calypogeia* and those of the ferns *Trichomanes bauerianum* Endl., *T. cuspidatum* Willd., and *Vittaria* spp. produce contaminant-free cultures that grow and generate their own gemmae faster than *Trichomanes speciosum* (Raine, 1994).

Moist, sterilized gemmae were not stored in the refrigerator during this study, but Dassler (pers. comm.) has observed healthy detached gemmae of *Callistopteris* on cultivated bryophyte mats collected two years earlier, which suggests that they may remain viable for considerably longer than dried spores of the Hymenophyllaceae. Wet storage of gemmae may be an effective way to increase the longevity of stored material of filmy ferns, providing a parallel with the improved longevity found for wet-stored spores of both green- and nongreen-spored ferns (Lindsay et al., 1992).

Once established, aseptic cultures generated from gemmae produce a potentially continuous and immortal supply of material, as they can simply be inverted over fresh dishes of media in a laminar flow unit and tapped to release



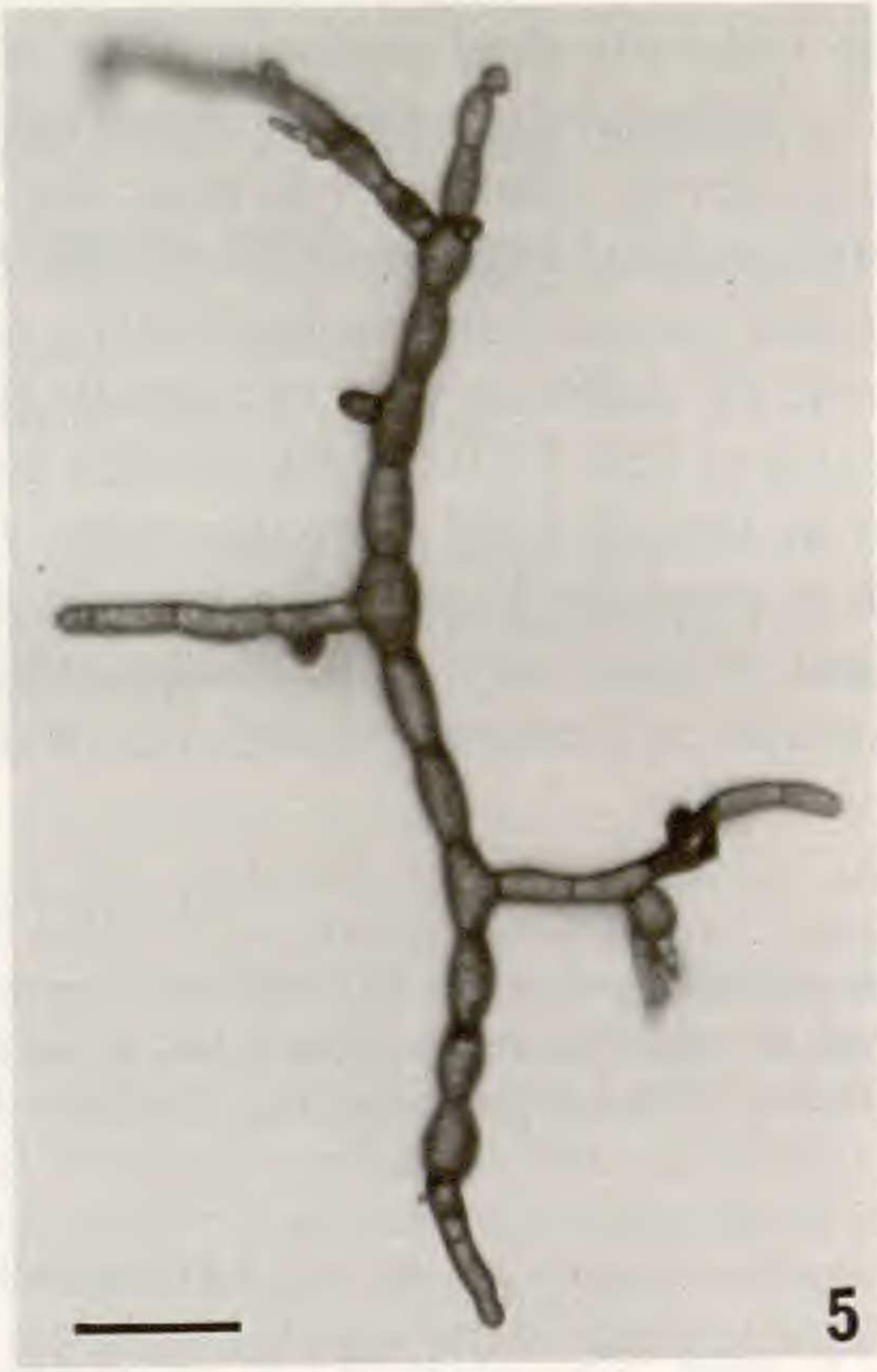


FIG. 4. Germinated gemma of *Trichomanes speciosum* in aseptic culture. Arrow indicates dehiscence scar of original gemma. Bar=100 μm.

FIG. 5. As in Fig. 4, but several weeks older; rhizoids and the characteristic three dimensional mat have started to form. Bar=100 μm.

FIG. 6. More than two year old aseptic culture of *Trichomanes speciosum* initiated from gemmae showing mature form of mat. Arrow indicates gemma production. Bar=300 μm.

gemmae onto the new surfaces. This method therefore provides the first opportunity for experiments involving fully defined media and conditions using this endangered species. It is now, for example, possible to test a vast range of environmental regimes and media supplements for their influence upon growth and gametangial induction.

Growth of cultures was extremely poor when dishes were maintained at temperatures and PAR levels similar to those recorded for *Trichomanes speciosum* in British field sites (Rumsey, 1994). This implies that growth of this species is severely limited by current climatic conditions in Britain, that recruitment of new sites is probably infrequent, and that sites with large areas of gametophyte cover represent many decades of growth.

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